

Isolation of Purified Allergens (Cod) by Isoelectric Focusing

S. ELSAYED and K. AAS

Department of Biochemistry, University of Oslo, Pediatric Department and Pediatric Research Institute, Rikshospitalet, University of Oslo, Oslo

Introduction

A chemically homogeneous allergen is a prerequisite for the study of the basic mechanism of allergic reactions. Allergens isolated from fish myogens have been shown to be convenient for model studies of the characteristics of the allergenic proteins [4-6]. The isolation of a major allergen designated DS 22, from the white muscle myogen of cod (*Gadus callarias*) has been reported [5]. Some of the chemical characteristics and immunological properties of this allergenic protein were recently studied [9]. On 7.5% acrylamide disc electrophoresis at alkaline pH fraction DS 22 gave three fast migrating zones while at acidic pH it gave one [5, 6]. Double diffusion-in-gel experiments with one rabbit specific antiserum to cod showed four precipitation lines occurring in two pairs. In the present study fraction DS 22 was further separated into allergenic and non-allergenic constituents.

Material and Methods

Fraction DS 22 was prepared from cod (*Gadus callarias*) white muscle tissue [4, 5]. An electrofocusing column of 110 ml capacity LKB 8101, a power supply LKB 3371 and ampholine carrier ampholytes covering the pH range 3-10 and 4-6 were supplied by LKB Produkter AB, Stockholm, Sweden.

The isoelectric focusing technique described by VESTERBERG and SVENSSON [19] was followed. Pilot experiments were performed to find out the most suitable experimental conditions and the desired pH range of the ampholytes. Different concentrations of urea or dimethyl formamide as solvents were tried and 8 M urea was finally used. Carrier ampholytes of pH range 4-6 were used in a concentration of 2 g%. 20 µg of DS 22 were dissolved in one of the fractions which was located approximately in the middle of the iso-

electric fractionation column. The experiment took 60 h and was performed at +4°. When the electrofocusing was terminated the zones of the allergen subcomponents were easily seen in visual light by the refraction difference between them and the rest of the column medium. Fractionation was performed with a flow rate 1-2 ml according to the location of the peaks. The pH of each fraction and its UV absorption at 280 nm in quartz cuvetts of 10 mm light path length, were recorded. The zones were collected and dialysed against running deionized water for 48 h. To assure the separation of the ampholytes from the allergenic proteins the dialysis bags were introduced into a glass electroanalysis column 800 × 45 mm. A continuous flow of 10 l fresh phosphate buffer (0.1 M and pH 7.4) was maintained during the 6-hour dialysis time. A d.c. potential of 1,000 V was applied between the ends of the column. When the dialysis was completed the fractions were lyophilized and stored at -20° for further investigations. The major fraction separated was re-run on the isoelectric focusing column using the same experimental conditions. The isoelectric location of this fraction was re-estimated.

Disc electrophoresis was performed in 7.5% acrylamide gel according to DAVIS [8]. 200-500 µg were dissolved in 200-400 µl of 6% sucrose solution and applied on the acrylamide discs. Mixtures of some of the separated fractions were also applied.

Specific antisera to cod were raised in rabbits and collected as described elsewhere [2]. Immunodiffusion was performed as outlined before [1] by the method of OUCHTERLONY [16], in a 1 mg/ml protein concentration of the different fractions.

Immuno-electrophoresis was done according to the method of GRABAR and WILLIAMS [12] utilising the micro-technique described by SCHEIDEGGER [18] with the modifications outlined by HIRSCHFELD [14]. The LKB outfit was utilised. The rest of the equipment and reagents were supplied as outlined in preceding papers [5, 6].

Allergic activity of the fractions was studied by means of direct skin testing on cod allergic patients and controls, and with passive transfer tests as described before [2, 4, 5, 6]. Before biological activity tests were performed the material was sterilized by Millipore filtration [2] and dilutions were made with 0.9% NaCl solution containing 0.5% phenol to a final concentration of 2 mg% of the respective material. For the present study the material was tested in 6 patients shown to be allergic to cod and possessing skin test reactivity to the allergens eliciting the allergic symptoms [5]. The material obtained from 4 different separation experiments was also used in skin tests on 6 control patients, not allergic to cod, but possessing skin reactivity to other allergens. Oral challenges on fish hypersensitive patients, were not performed in this experiment because previous challenges of the cod hypersensitive patients with DS 22 have elicited so fierce reactions that further provocations were considered unwarranted [5] and good correlation has been established between clinical hypersensitivity to cod and positive skin test reactions to dilute cod extract in the fish allergic patients [3].

Results

Allergen Fractionation

The trials performed to estimate the most suitable pH range of the ampholyte, by using a wide pH range (3-10), gave two fractions having isoelectric points of 3.70 and 4.90. With use of the ampholyte of pH range 4-6, five minor fractions and a major one were isolated from fraction DS 22 and were characterized by their pI values (table I). The calculated pI values were an average of 5 identical

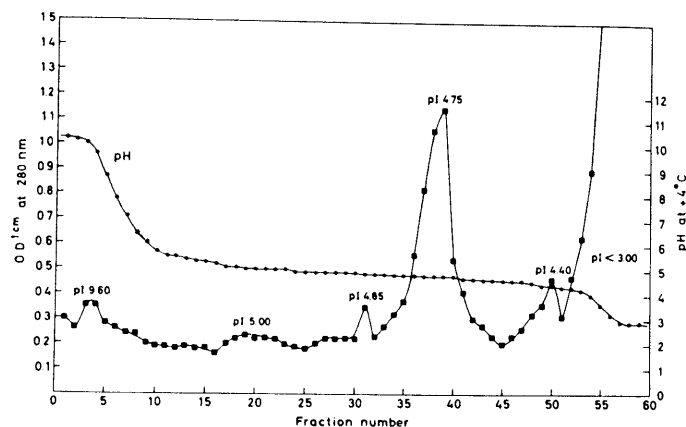


Fig. 1. The isoelectric fractionation of DS 22.

Table I. pI values of the constituents of DS 22 and their percentage of the total yield

Mean pI value $\pm$ standard deviation	Percent of the total weight	Designation
9.60 ± 0.10	6.18	Fr. pI 9.60
5.00 ± 0.05	20.90	Fr. pI 5.00
4.85 ± 0.04	1.35	Fr. pI 4.85
4.75 ± 0.03	50.71	Fr. pI 4.75
4.40 ± 0.01	10.19	Fr. pI 4.40
< 3.00	10.76	Fr. pI < 3.00

experiments. A graph for the OD at 280 nm and pH gradient versus the fraction number was plotted from one of the trials (fig. 1). The effect of change of temperature from $+2$ to $+13^\circ\text{C}$ was investigated in 6 experiments and was found of negligible influence on the estimated pI values. Fr. pI < 3.00 migrated into the anode solution, therefore, it was difficult to determine its isoelectric point precisely. After re-run of Fr. pI 4.75 on the isoelectric focusing column, this fraction reappeared as one single zone at the same isoelectric point as before (fig. 2).

On 7.5% acrylamide disc electrophoresis at pH 8.3 each of the sub-components gave one single band, except in the case of Fr. pI 4.40 in

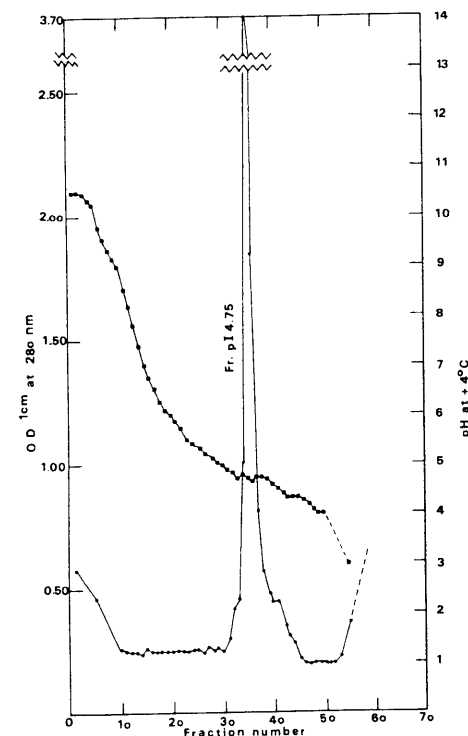


Fig. 2. The isoelectric focusing rerun of Fr. pI 4.75 under identical conditions to those used for DS 22 separation.

which two more slowly migrating contaminant bands were observed in the gel discs. The run from one batch is illustrated in figure 3. Mixtures of two (Fr. pI 4.85, Fr. pI 4.75) and three (Fr. pI 4.85, Fr. pI 4.75, Fr. pI 4.40) of the fractions gave two and three bands on the acrylamide discs, respectively.

The double diffusion-in-gel analyses showed the presence of some common antigenic determinants in Fr. pI 4.75 (fig. 4 and 5) as in DS 22.

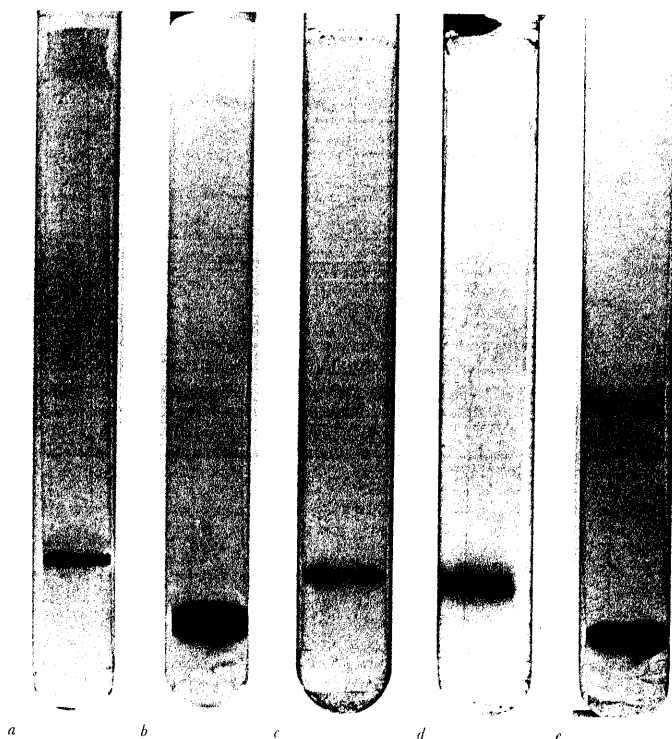


Fig. 3. Disc electrophoresis of the isoelectric focusing constituents from one batch. *a* Fr. pI 9.60, *b* Fr. pI 5.00, *c* Fr. pI 4.85, *d* Fr. pI 4.75, *e* Fr. pI 4.40.

Immuno-electrophoresis experiments showed that the pI 4.75 material migrated as one band with homogenous mobility but with three distinct precipitation lines developed against the rabbit antiserum to cod.

Allergenic activity Fr. pI 4.75 and Fr. pI 4.85 were the most active, eliciting marked positive skin reactions in all of the cod allergic individuals (table II and III). The degree of the activity was of the same extent as that of the parent substance DS 22. Some activity was present also in Fr. pI 4.40, but less than that found in the two active

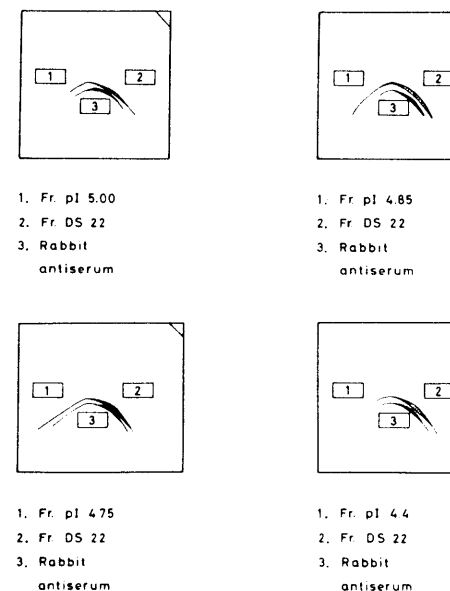


Fig. 4. Schematic illustration of the results of double diffusion-in-gel of the isoelectric focusing fractionation products.

ones. Fr. pI 9.60 and Fr. pI 5.00 were inactive in the cod allergic individuals eliciting only slight irritative reactions in a few instances. Negative or only minute irritative skin test reactions were elicited by the materials on the control individuals.

Two passive transfer experiments with DS 22 and with Fr. pI 4.85 and Fr. pI 4.75 as challenging allergens confirmed that the two sub-components were highly active.

All three materials used at 0.2 mg/100 ml concentrations elicited very marked wheal and flare (++++) reactions in normal skin sensitized with serum of cod allergic donor. Comparative quantitation showed that Fr. pI 4.75 contained an allergenic activity of the same order as that of DS 22 within the limits of this method. Both material showed end points activities at $2 \cdot 10^{-14}$ mg/ml in one series of passive transfer tests and $2 \cdot 10^{-16}$ mg/ml in a second series, with the serum and

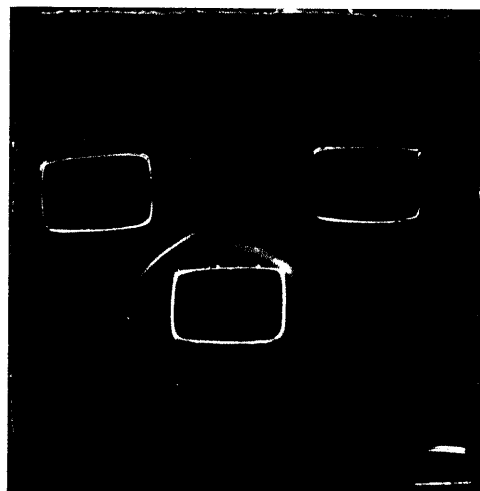


Fig. 5. Double diffusion-in-gel of Fr. pI 4.75. Left well: Fr. pI 4.75. Right well: DS 22. Middle well: Specific antiserum.

recipient used. No reaction occurred in the sites sensitized with control serum. Sites on the volar surfaces of the forearms of a non-allergic volunteer were sensitized with serum from the cod-allergic donor (who tolerates beef) and a control individual sensitive to allergens other than fish. Approximately 24 h later, the recipient ate 2 mg of fraction pI 4.75 with approximately 100 g of pure beef. Within the next 30 minutes, urticarial wheals occurred at all sites sensitized with the fish-allergic sera but not in the control serum sites.

Passive transfer cross neutralization experiments showed that Fr. pI 4.85 could completely exhaust the reactivity of the sites towards both DS 22 and Fr. pI 4.75 and vice versa, indicating that the allergenic determinants(s) of the two fractions and DS 22 are identical or completely cross-reacting.

Discussion

The separation of DS 22 into several constituent fractions was based on difference in their isoelectric points. Although the difference

Table II. Biological activity of the constituents of DS 22 tested in 6 patients

Material	Patient initials					
	P. H. H.	P. W.	V. H.	G. B. G.	A. A.	L. A. G.
0.1% histamine	+++	+++	+++	+++	+++	+++
Control solution	-	-	-	-	-	-
DS 22	+++++	+++++	+++++	+++++	+++++	+++++
Fr. pI 9.60	+	-	-	-	+	-
Fr. pI 5.00	+	-	-	+	+	-
Fr. pI 4.85	+++++	+++++	+++++	+++++	+++++	+++++
Fr. pI 4.75	+++++	+++++	+++++	+++++	+++++	+++++
Fr. pI 4.40	+++++	+++	+++	+++++	++	+++++

Table III. Biological activity of the fractions from different preparations tested in one patient (A.A.)

Number of preparations	16/68	18/68	22/68	24/68
Fr. pI 9.60	+	-	-	-
Fr. pI 5.00	+	-	-	+
Fr. pI 4.85	++++	++++	++++	++++
Fr. pI 4.75	+++++	++++	+++++	+++++
Fr. pI 4.40	++	++ (+)	+++	+++ (+)
0.1% Histamine	+++			
Control solution	-			

between some of the fractions was small, a complete separation was achieved by using a slow flow rate during elution.

The carrier ampholyte concentration had to be raised to 2 g% to avoid the precipitation of the focused protein zones at their isoelectric points. This increase had no effect on the pI values obtained [19]. The nomenclature used is valid only for fractions separated under the standard conditions defined. The electrodialysis and the prolonged continuous-flow dialysis were utilised to assure the separation of the carrier ampholytes from the purified fractions.

The effect of urea on fish myogen denaturation has been investigated by Konosu *et al.* [15] showing that the viscosity and crystallization properties of the myogen 'Component 3' from carp are unchanged after treatment for 4 h in 8 M urea at room temperature. Fraction DS 22 retains its allergenic activity, and apparently most of its native conformation after dialysis against large volumes of distilled water for 24 h, subsequent to treatment with high concentration of guanidine

hydrochloride or 8 M urea at $+4^\circ$ for 24 h [10]. Acrylamide disc electrophoresis of the parent material DS 22 at acidic pH, has previously given one single band [5], therefore, it was found unnecessary to repeat it at the same pH values with the constituents. The main constituents separated have shown homogeneity on disc electrophoresis at alkaline pH. Each has given one single, intense and fast migrating zone (fig.3). Chemically, Fr. pI 4.75 belongs to the most purified allergenic proteins reported as indicated by its homogeneity in immunoelectrophoretic mobility, in molecular sieving on Sephadex columns [5], and in ultracentrifugation [11]. If this fraction contains contaminants they represent a very small proportion of the whole preparation. The mixtures of Fr. pI 4.85, Fr. pI 4.75 and Fr. pI 4.85, Fr. pI 4.75, Fr. pI 4.40 gave two and three bands respectively on the acrylamide discs. Each of these bands corresponded to one of the fractions which migrated separately due to different charges. Fr. pI 4.75 re-run on the isoelectric focusing column, was isolated as one distinct zone located approximately in the middle of the column at the same isoelectric point as before.

With antisera obtained from 11 rabbits of the same species only one precipitation line was formed with DS 22 in immunodiffusion-in-gel experiments. However, with an antiserum obtained from another similar rabbit identically immunized, both DS 22 and Fr. pI 4.75 generated more than one precipitation line. These lines may represent molecular variants of the same protein or two or more antigenic determinants on different localization of the same molecule, reacting with distinct antibodies in different zones of equilibrium [7, 13]. The immunoelectrophoretic mobility of Fr. pI 4.75 was homogeneous, indicating a mono-covalent structure of the material in accordance with the other chemical parameters discussed. However, this material probably possesses three antigenic determinants shown by three precipitation lines. Other antigenic determinants may also exist on the same molecule without giving precipitation in the immunodiffusion experiments [13].

A protein molecule is a mosaic of individually specific antigenic determinants at different sites of the molecular chain. The demonstration of immunological heterogeneity does not necessarily contradict with the biochemical purity. It has been claimed that highly purified proteins when allowed to diffuse against their antibodies in-gel would form *one primary* precipitation band which then will act like a barrier preventing either reactants to pass through [17]. Presupposition for

this is, however, that the two reactants are used in their optimum equivalent concentrations and under strict ideal conditions, otherwise the precipitating band might split into more than one [7]. Whether one protein molecule may give more than one precipitation line or not, in immunodiffusion-in-gel experiments, is subject of discussion. Two opposing hypotheses exist as reviewed by RAYNAUD [17].

As great differences are found between animals of the same species, as to the capacity to form precipitating antibodies against the same antigenic material, a large number of rabbits has to be immunized to test either hypotheses.

The assay of the allergenic activity by skin testing has shown that three of the fractions isolated were active. It has previously been shown that DS 22 contains the major allergen of cod which is responsible for most of the allergic reactions observed when cod is eaten, when cod emanations are inhaled or when cod extracts are used in skin tests and passive transfer experiments [5]. Passive transfer cross neutralization tests showed that fraction pI 4.75 and the parent allergenic material DS 22 contained the same or completely identical allergenic determinants. The specificity of the reaginic serum used, has been demonstrated in preceding work [5]. Fr. pI 4.75 which is the main purified component, can therefore be considered representative of the major allergenic determinant(s) of cod. At the same time, it appears to be the most potent allergenic protein available with regards to the allergenic activity end points found in two series of the passive transfer experiments.

The myogens of fish are not represented among mammalian proteins. Their amino acid composition and their strongly acidic characters are different from those of human proteins [9]. Such proteins may easily be recognized as 'foreign' by the immune system of human individuals, accounting for the relatively frequent occurrence of immunological hypersensitivity in a fish-eating population [5].

Summary

Further purification of a partially purified cod allergen (DS 22), by isoelectric fractionation resulted in the isolation of one major (Fr. pI 4.75) and 5 minor subconstituents designated according to their isoelectric points. Fraction pI 4.75 contained the highest yield and allergenic activity compared to the other fractions and was found to be representative of the activity of DS 22.

References

1. AAS, K.: Studies of hypersensitivity to fish. Preparation and characterization of extracts from cod and salmon. *Int. Arch. Allergy* 29: 453-469 (1966).
2. AAS, K.: Studies of hypersensitivity to fish. Studies of some immunochemical characteristics of allergenic components of fish extract (cod). *Int. Arch. Allergy* 29: 536-552 (1966).
3. AAS, K.: Studies of hypersensitivity to fish. Characterization of different allergen extracts from fish with respect to content of protein antigens and allergenic activity. *Int. Arch. Allergy* 30: 1-14 (1966).
4. AAS, K.: Studies of hypersensitivity to fish. Studies of different fractions of extracts from cod muscle tissue. *Int. Arch. Allergy* 31: 239-260 (1967).
5. AAS, K. and JEBSEN, J. W.: Studies of hypersensitivity to fish. Partial purification and crystallization of a major allergenic component of cod. *Int. Arch. Allergy* 32: 1-20 (1967).
6. AAS, K. and ELSAYED, S. M.: Characterization of a major allergen (cod). Effect of enzymic hydrolysis on the allergenic activity. *J. Allergy* 44: 333-343 (1969).
7. CROWLE, A. J.: Immunodiffusion, pp. 53, 72 (Academic Press, London/New York 1961).
8. DAVIS, B. J.: Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* 121: 404-427 (1964).
9. ELSAYED, S. M. and AAS, K.: Characterization of a major allergen (cod). Chemical composition and immunological properties. *Int. Arch. Allergy* 38: 536-548 (1970).
10. ELSAYED, S. and AAS, K.: Characterization of a major allergen (cod). Observations on effect of denaturation on the allergenic activity (in preparation).
11. ELSAYED, S.; AAS, K., and CHRISTENSEN, T.: Partial characterization of homogeneous allergens (cod). *Int. Arch. Allergy* 40: 439-447 (1971).
12. GRABAR, P. et WILLIAMS, C. A., jr.: Methode permettant l'étude conjuguée des propriétés électrophorétiques et immunochimiques d'un mélange de protéines, application au serum sanguin. *Biochim. biophys. Acta* 10: 193-194 (1953).
13. HARBOE, M.; SOLHEIM, B. G., and DEVERILL, J.: Individual antigenic specificity of human macroglobulins. Localization of antigenic determinants and their use to demonstrate formation of hybrid molecules after reduction and reassociation. *J. exp. Med.* 129: 1217/1233 (1969).
14. HIRSCHFELD, J.: Immunoelectrophoresis. Procedure and application to the study of group-specific variations in sera. *Sci. Tools* 7: 18-25 (1960).
15. KONOSU, S.; HAMOIR, G., and PÉCHÈRE, J. F.: Carp myogens of white and red muscle. Properties and amino acid composition of the main low-molecular weight components of white muscles. *Biochem. J.* 96: 98-112 (1965).
16. OUCHTERLONY, O.: Diffusion-in-gel methods for immunological analysis. *Progr. Allergy*, vol. 5, pp. 1-78 (Karger, Basel 1958).
17. RAYNAUD, M.: Heterogeneity of diphtheria antibodies. *Proc. 2nd Meet. Fed. europ. Biochem. Soc., Vienna 1965*, pp. 197-251 (Pergman Press, Oxford/New York 1966).
18. SCHEIDEGGER, J. J.: Une micro-méthode de l'immuno-électrophorèse. *Int. Arch. Allergy* 7: 103-110 (1955).
19. VESTERBERG, O. and SVENSSON, H.: Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. IV. Further studies on the resolving power in connection with separation of myoglobins. *Acta chem. scand.* 20: 820-834 (1966).

Manuscript received: August 5, 1970.

Authors' address: S. ELSAYED and Prof. K. AAS, Department of Pediatrics, Rikshospitalet, University Hospital, Oslo (Norway)

Partial Characterization of Homogeneous Allergens (Cod)

S. ELSAYED, K. AAS and T. CHRISTENSEN

Pediatric Research Institute, Pediatric Department, Rikshospitalet, and Department of Biochemistry, University of Oslo, Oslo

The isolation and crystallization of a protein fraction (DS 22) containing the major allergen(s) in cod white muscle myogen has been reported [1, 2]. This fraction was found to contain small amounts of monosaccharides. The amino acid analysis of this fraction revealed peculiar composition of the aromatic amino acids with a high proportion of phenyl alanine residues and a low proportion of tryptophan and tyrosine residues. The molecular weight of DS 22 was estimated to be 14,500 by sedimentation and diffusion methods [10]. The effect of enzymatic degradation on the biological allergenic activity was also reported [3]. Further purification of DS 22 by isoelectric focusing on carrier ampholytes in a sucrose density gradient resulted in the isolation of one major and 5 minor constituents which were designated according to their estimated isoelectric points. The allergenic activity and antigenicity of the subcomponents were investigated and an allergenic identity was demonstrated between Fr. pI 4.75 and DS 22 [11]. Fr. pI 4.75 was shown to contain the major allergenic determinant(s) of cod and was considered a suitable representative for model studies of the allergenic proteins.

The results given below present the chemical composition, the UV absorption characteristics and the sedimentation analysis for Fr. pI 4.75 and for some of the other polypeptides separated by the isoelectric fractionation technique.

Material and Methods

Fr. pI 4.75 was isolated from the white muscle myogen of cod (*Gadus callarias*) as outlined elsewhere [1, 2, 11]. A ratio-recording spectrophotometer, Beckman DK. 2A was used for UV spectra scanning. Quantitative amino acid analyzer, Beckman Unichrome was utilized